Attorney Docket # 82402-3802

EXAMINER	Suryaprabha Chunduru
GROUP	1656
APPLICANT	Guy et al.
SERIAL NO:	09/720,206
FILED	May 3, 2001
FOR	NONSYMBIOTIC PLANT HEMOGLOBINS TO MAINTAIN CELL ENERGY STATUS

Commissioner of Patents
Washington, D.C., 20231
U.S.A.

Dear Sir:

DECLARATION

I, the undersigned, am Dr. Robert Hill, (Professor, Department of Plant Science, University of Manitoba). I am also one of the inventors of the above-referenced application.

Plant hemoglobins have been classified into symbiotic and nonsymbiotic types: symbiotic hemoglobins are found in plants that are capable of participating in microbial symbioses, where they function in regulating oxygen supply to nitrogen fixing bacteria; nonsymbiotic hemoglobins were thought to be the evolutionary predecessors of the more specialized symbiotic leghemoglobins. As discussed below, these two classes of proteins have very different oxygen binding characteristics and functions, and these distinctions are understood by individuals in the field.

Previously, very little was known about the function of Hb, although it had been proposed that nonsymbiotic hemoglobins may act either as oxygen carriers to facilitate oxygen diffusion, or oxygen sensors to regulate expression of anaerobic proteins during periods of low oxygen supply. As discussed in the application as filed, nonsymbiotic plant hemoglobins have been shown to have

high oxygen avidity, with dissociation constants for oxyhemoglobin of 2.86 nM, 0.55 nM and 1.6 nM respectively, resulting in conditions whereby the free protein will remain oxygenated at oxygen concentrations far below those at which anaerobic processes are activated. Thus, while roles for Hb in the facilitated diffusion and sensing of oxygen had been proposed, it was unlikely that the nonsymbiotic plant hemoglobins would function as either facilitators of oxygen diffusion or sensors of oxygen. Thus, while Hb or Hb related proteins are found in all divisions of living organisms, their function had not been well defined.

Furthermore the dissociation constant of barley oxyhemoglobin iindicates that oxyhemoglobin, acting alone, would be ineffective in providing oxygen to maintain mitochondrial respiratory processes. This was confirmed by the observation that Antimycin A has no effect on the ability of hemoglobin-containing cells in maintaining their energy status under low oxygen tensions.

As discussed in the instant application, it was only once we grew the plants under nitrogen that the effect of the nonsymbiotic hemoglobins on ATP levels was noted. That is, this was when it was discovered that the nonsymbiotic plant hemoglobins are involved in the binding of oxygen under low oxygen environments and thereby maintaining cell energy status. This in turn resulted in the discovery of potential uses for overexpressing nonsymbiotic plant hemoglobins. The use of nonsymbiotic plant hemoglobins to maintain cell energy status in low oxygen environments is not taught or suggested in the prior art.

Thus, the instant application demonstrates that nonsymbiotic hemoglobins function to maintain the energy status of cells exposed to low oxygen tensions. That is, the results in the application as filed show clearly that the energy status of cells when oxygen is limiting is affected by the ability of the cells to produce hemoglobin. Total adenylates and ATP levels are maintained during the period of exposure to limiting oxygen when hemoglobin is constitutively expressed in the cells. Furthermore, when hemoglobin expression is suppressed by constitutive expression of antisense barley hemoglobin message, the cells are unable to maintain their energy status during oxygen limitation.

Bailey describes the importance of increased intracellular oxygen levels as the functional mode of action of the *Vitreoscilla* hemoglobin (see for example, Bailey page 4, lines 6 and 11 and page 6, lines 19, 23 and 27). Bailey also notes the particular suitability of a hemoglobin with high k_{off} rates or low oxygen affinity (page 7, lines 5-10). As discussed in the application as filed, in the previous response and above, the nonsymbiotic plant hemoglobins have low k_{off} rates. Furthermore, the nonsymbiotic hemoglobins have high oxygen avidity, not low oxygen affinity as is the case with horse heart myoglobin, another target listed by Bailey as "particularly suitable". Thus, Bailey teaches against the use of nonsymbiotic plant hemoglobins as these proteins have very different properties, specifically, vastly different oxygen binding characteristics compared to those described by Bailey and are clearly not functionally equivalent. Furthermore, based on the properties of the nonsymbiotic hemoglobins, one of skill in the art, in view of Bailey, might conclude that overexpression of nonsymbiotic hemoglobin proteins would in fact restrict oxygen availability in a cell rather than increase it and would therefore have a negative impact on agronomic properties of a plant.

Regarding Andersson, Andersson only proposes multiple functions for nonsymbiotic plant hemoglobins. These include acting as a sensor of oxygen concentration (paragraph 1, line 3), acting as a facilitator of oxygen diffusion at low oxygen concentrations (paragraph 1, lines 12-13), acting in oxygen transport (paragraph 1, line 14), acting as a facilitator of oxygen diffusion in dividing cells(paragraph 2, lines 2-6), being associated with high levels of metabolic activity (paragraph 2, lines 6-10), and facilitating intracellular diffusion of oxygen to the mitochondria (paragraph 2, lines 15-18). As discussed above, it was shown that over-expression of the nonsymbiotic hemoglobins had no effect on oxygen diffusion or on oxidative respiration which would have led one to conclude that Bailey was right and the nonsymbiotic hemoglobins were not functional or were of no benefit when overexpressed in plants.

In summary, while many possible roles had been previously hypothesized for nonsymbiotic plant hemoglobins such as an oxygen sensor,

facilitator of oxygen diffusion, and maintaining mitochondrial respiration, to nam a few, th se proposed functions w re found to be incorrect when examined experimentally or were shown to be unlikely in view of or inconsistent with the physical characteristics of the nonsymbiotic plant hemoglobins. As discussed above, it was only once we grew the plants under nitrogen that the effect of the nonsymbiotic hemoglobins on ATP levels was noted and their potential utility was understood.

I declare that all statements made therein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the instant patent application or any patent issuing therefrom.

SIGNED at Winnipeg
(place of execution)

this 10th day of December, 2003

Robert D. Hill

Robert Hill